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Characterization of the Bovine Innate Immune Response to Intramammary Infection with *Klebsiella pneumoniae*

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ABSTRACT

Gram-negative bacteria are responsible for almost one-half of the clinical cases of mastitis that occur annually. Of those gram-negative bacteria that induce mastitis, *Klebsiella pneumoniae* remains one of the most prevalent. Detection of infectious pathogens and the induction of a proinflammatory response are critical components of host innate immunity. The objective of the current study was to characterize several elements of the bovine innate immune response to intramammary infection with *Klebsiella pneumoniae*. The inflammatory cytokine response and changes in the levels of soluble CD14 (sCD14) and lipopolysaccharide (LPS)-binding protein (LBP), 2 proteins that contribute to host recognition of gram-negative bacteria, were studied. The contralateral quarters of 7 late-lactating Holstein cows were challenged with either saline or *K. pneumoniae*, and milk and blood samples were collected. Initial increases in the chemoattractants C5a and IL-8, as well as TNF-α, were evident in infected quarters within 16 h of challenge and were temporally coincident with increases in milk somatic cells. Augmented levels of TNF-α and IL-8 were observed in infected quarters until >48 h postchallenge, respectively. Elevated levels of IL-12, IFN-γ, and the antiinflammatory cytokine, IL-10, which were first detected between 12 and 20 h postinfection, persisted in infected quarters throughout the study (>96 h). Initial increases in milk LBP and sCD14 were detected 16 and 20 h, respectively, after challenge. Together, these data demonstrate that intramammary infection with *K. pneumoniae* elicits a host response characterized by the induction of proinflammatory cytokines and elevation of accessory molecules involved in LPS recognition.
(**Key words:** lipopolysaccharide-binding protein, *Klebsiella pneumoniae*, mastitis, innate immunity)

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Abbreviation key: HRP = horseradish peroxidase, **LBP** = LPS-binding protein, **mCD14** = membrane-bound CD14, **OD** = optical density, **sCD14** = soluble CD14, **TBS** = Tris-buffered saline, **TMB** = tetramethylbenzidine, **Tlr** = toll-like receptor.

INTRODUCTION

Mastitis remains one of the most costly diseases to animal agriculture throughout much of the world (National Mastitis Council, 1999). Although implementation of effective control programs has significantly reduced the incidence of subclinical mastitis caused by such contagious pathogens as *Staphylococcus aureus* and *Streptococcus* spp., these programs alone have been generally ineffective at preventing IMI with Gram-negative pathogens (Erskine et al., 1991). Approximately 40% of the clinical cases of mastitis that occur annually are caused by gram-negative bacteria, and nearly 25% of those cows with severe gram-negative mastitis infections will either die or be culled (Eberhart, 1984; Erskine et al., 1991). Because the incidence of clinical mastitis arising from gram-negative bacterial infection is inversely proportional to bulk milk SCC (Barkema et al., 1998), economic losses attributed to intramammary gram-negative infection can be expected to increase as dairymen continue to strive for lower bulk milk SCC. A further complication associated with coliform mastitis is that current antimicrobial therapy for treatment remains suboptimal (Erskine et al., 1991; Ziv, 1992).

Escherichia coli, various species of *Enterobacter*, and *Klebsiella pneumoniae* constitute the most common pathogens implicated in coliform mastitis (Smith et al., 1985). Mastitis resulting from *Klebsiella* infection is often severe and can result in markedly decreased milk production (Newman, 1975). The mammary glands of cows that have died as a result of *Klebsiella* mastitis show signs of massive inflammation and widespread tissue necrosis. Interestingly, much of our understanding of the innate immune response to gram-negative infection comes from studies using *E. coli* as the organism of choice for bovine IMI (Shuster et al., 1995; Shuster et al., 1997; Hirvonen et al., 1999; Lee et al., 2003).

In contrast, much less is known about the mammary gland innate immune response to bacteria of the genus *Klebsiella*. Several differences in the pathogenicity of *E. coli* and *Klebsiella* spp. during mastitis have been reported, including: 1) growth of *E. coli* is inhibited to a greater extent than that of *K. pneumoniae* by secretions from nonlactating glands (Todhunter et al., 1990); 2) the majority of IMI resulting from *Klebsiella* originate during lactation, whereas those arising from *E. coli* tend to occur during the nonlactating period (Todhunter et al., 1991); and 3) IMI caused by *Klebisella* have a longer duration than those resulting from *E. coli* (Todhunter et al., 1991). Together, these data establish clear differences between the pathogenicity of *E. coli* and *Klebsiella* spp. during mammary gland infection.

The innate immune response is characterized by the rapid activation of antimicrobial host defense mechanisms that are able to respond to a wide array of pathogens (Suffredini et al., 1999). The ability to respond to a vast number of pathogens is mediated by the expression of evolutionarily conserved pattern recognition receptors that are capable of recognizing common bacterial motifs shared by diverse pathogens. Toll-like receptor (**Tlr**)-4, which is expressed on a wide array of cell types including macrophages, neutrophils, and epithelial cells, is one such pattern recognition receptor (Akira et al., 2001). This receptor recognizes bacterial LPS, a component of the outer leaflet of all gram-negative bacteria including *Klebsiella*. The Tlr-4 recognition of LPS is facilitated by the accessory molecules LPS-binding protein (**LBP**) and CD14. During infection, the acute-phase protein LBP binds LPS and facilitates the transfer of LPS to membrane-associated CD14 (**mCD14**) found on cells of monocytic lineage and neutrophils (Schumann et al., 1990; Wright et al., 1990). The resulting LPS-mCD14 complexes associate with Tlr-4 leading to cellular activation (Akira et al., 2001). The LBP also facilitates the transfer of LPS to soluble CD14 (**sCD14**) and the resultant LPS-sCD14 complexes can then activate Tlr-4 signaling on cells that lack mCD14, such as endothelial and epithelial cells (Tapping and Tobias, 1997).

Cellular activation by Tlr-4 and other pattern recognition receptors leads to the generation of an inflammatory response that is elicited, in part, by cytokine production (Dinarello, 1996; Koj, 1996; Suffredini et al., 1999). Proinflammatory cytokines, such as TNF-α and IL-1β, are potent inducers of the acute-phase response, fever, and vascular endothelial activation, the latter of which facilitates neutrophil migration to the site of infection (Thijs et al., 1996; Dinarello, 1996). The IL-8 is a chemoattractant that recruits circulating neutrophils to the site of infection (Harada et al., 1994). Other cytokines function to link the innate and adaptive branches of immunity. The IL-12 contributes to the innate immune response by stimulating the production of IFN-γ, an activator of neutrophils and macrophages (Trinchieri, 1997). In addition, IL-12 stimulates proliferation and induces the differentiation of T lymphocytes, thus, contributing to the adaptive immune response (Ma, 2001). Finally, cytokines, such as IL-10, contribute to the resolution of inflammation by inhibiting macrophage proinflammatory cytokine production (Spits and de Waal Malefyt, 1992; Redpath et al., 2001).

Because little is known about the mammary gland innate immune response to *K. pneumoniae*, we decided to characterize bovine mammary gland cytokine expression following IMI with this organism. In addition, we studied changes in the expression of LBP and sCD14, 2 accessory molecules that contribute to host cell recognition of *K. pneumoniae* by binding LPS and facilitating its presentation to Tlr-4.

MATERIALS AND METHODS

Cows

Seven healthy, late-lactating Holstein cows (303.8 ± 42.9 DIM) were selected on the basis of milk SCC of <500,000 cells/mL and the absence of detectable bacteria growth from 3 daily consecutive aseptic milk samples plated on blood agar plates. Mean (+SE) milk SCC for all quarter just prior to challenge was 119,500 + 35,000. To quantitate somatic cells, milk samples were heated to 60°C and subsequently maintained at 40°C until counted on an automated cell counter (Fossomatic model 90, Foss Food Technology, Hillerød, Denmark) as previously described (Miller et al., 1986).

Intramammary Challenge with *K. pneumoniae*

Prior to intramammary challenge, 10 mL of brain heart infusion broth (Becton-Dickinson Diagnostic Systems, Inc., Sparks, MD) was inoculated with a frozen strain of *K. pneumoniae* and incubated for 18 h at 37°C. The *K. pneumoniae* strain 2612 used in this study had been previously isolated from a clinical case of mastitis (gift of E. J. Carroll, University of California, Davis). The resulting broth was streaked onto a trypticase soy agar (Becton-Dickinson Diagnostic Systems, Inc.) plate containing 5% bovine blood and 0.1% esculin. After incubating the plate overnight at 37°C, a single colony was transferred to 10 mL of trypticase soy broth (Becton-Dickinson Diagnostic Systems, Inc.) and incubated at 37°C for 20 h. The bacteria were centrifuged at 2000 × *g* for 10 min at 4°C, and the resulting pellet washed 3 times with sterile PBS (Cambrex Bio Science Walkersville, Inc., Walkersville, MD). After the final wash, the bacterial pellet was resuspended in 10 mL of sterile

PBS, and the suspension was diluted until a transmittance of 80% at 610 nm was obtained. This transmittance corresponded with a concentration of approximately 1×10^8 cfu/mL. A series of dilutions in PBS were performed to yield a final approximate concentration of 50 cfu/mL. Immediately following the morning milking, the left front or right rear quarters of each animal were infused with 2 mL of either the final dilution of *K. pneumoniae* or PBS alone, respectively. The actual number of bacteria injected (139 cfu) was confirmed using pour plates in which 1 mL of the final dilution of *K. pneumoniae* was added to 9 mL of melted MacConkey agar (Becton-Dickinson Diagnostic Systems, Inc.). The plates were allowed to cool and solidify at room temperature, and were subsequently incubated overnight at 37°C. Aseptic milk samples were collected from control and infected quarters throughout the study, plated, and sent to the Maryland Department of Agriculture Animal Health Section (College Park, MD) for confirmatory identification.

Whey and Plasma Preparation

For the preparation of whey, milk samples were centrifuged at 44,000 × *g* at 4°C for 30 min, and the fat layer was removed with a spatula. The skimmed milk was decanted into a clean tube and centrifuged again for 30 min as above, and the translucent supernatant was collected and stored at –70°C. Tail vein blood samples were collected in Vacutainer glass tubes containing K_3 EDTA (Becton-Dickinson Corp, Franklin, Lakes, NJ). For the determination of differential white blood cell counts, the freshly collected blood was inverted ×10, placed on a rocker for 15 min, and analyzed using a HEMAVET 3700 automated multi-species hematology system (CDC Technologies, Inc., Oxford, CT). For subsequent preparation of plasma, the freshly collected blood was inverted ×10 in K_3 EDTA glass tubes, centrifuged at 1500 × *g* for 15 min, and the clear plasma supernatant was collected, aliquoted, and stored at –70°C.

ELISA

Sandwich ELISA were used to quantify milk levels of the following: TNF-α, IL-1β, IL-10, and IL-12. For all of these ELISA, flat-bottom 96-well plates (Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with capture antibody diluted in 0.05 *M* sodium carbonate, pH 9.6. The plates were washed ×4 with 0.05% Tween 20 diluted in 50 m*M* Tris-buffered saline (TBS), pH 8.0, and subsequently blocked with 2% fish skin gelatin (Sigma Chemical Co., St. Louis, MO) for 1 h at room temperature. Plates were washed, and 100 μl of whey samples or serially diluted standards was added to each well. For several assays, dilution of the whey samples in wash buffer was necessary to insure that their values fell within the linear portion of the standard curve. Plates were incubated for 1 to 1.5 h at room temperature and were subsequently washed as above. Detection antibodies were diluted in TBS wash buffer containing 2% gelatin, and 100 μL of this solution was added to each well. Plates were incubated for 1 h at room temperature and washed as above. Anti-IgG antibodies (Becton-Dickinson Corp.) or streptavidin (Sigma Chemical Co.) conjugated to horseradish peroxidase (**HRP**) were diluted in TBS wash buffer containing 2% gelatin, and 100 μL of this solution was added to each well. Plates were incubated for 1 h at room temperature and washed, and 100 μL of 3,3′,5,5′-tetramethylbenzidine (**TMB**) substrate solution (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) was added to each well. The reaction was stopped by the addition of 100 μL of 2 *M* H_2SO_4, and the absorbance read at 450 nm on a microplate reader (Bio-Tec Instruments, Inc., Winooski, VT). A background correction reading at 565 nm was subtracted from the 450-nm absorbance readings.

ELISA for TNF-α

The ELISA used to measure milk levels of TNF-α has been previously described (Paape et al., 2002), and performed here with only slight modification. Ascites (2C4) containing mouse anti-recombinant bovine TNF-α antibodies diluted 1:1,000 were used as the capture antibody. Rabbit anti-recombinant bovine TNF-α polyclonal serum diluted 1:5,000 was used for detection of the captured TNF-α. Goat anti-rabbit IgG conjugated to HRP (Becton-Dickinson Corp.) diluted 1:5,000 was used for detection of the anti-TNF-α polyclonal serum antibodies. Whey samples were diluted 1:5 in wash buffer prior to assaying. Recombinant bovine TNF-α (Genentech Corp., South San Francisco, CA) was diluted to generate a standard curve. The concentration of TNF-α was calculated by extrapolating from a standard curve and expressed in nanograms per milliliter.

ELISA for IL-1β

The ELISA used to measure milk levels of IL-1β has been previously described and performed here with only slight modification (Riollet et al., 2000). Mouse anti-ovine IL-1β antibodies (clone 1D4; Serotec, Inc., Raleigh, NC) diluted to 5 μg/mL were used as the capture antibody. Rabbit anti-recombinant ovine IL-1β polyclonal serum diluted 1:500 was used for detection of the captured IL-1β. Goat anti-rabbit IgG conjugated

to HRP (Becton-Dickinson Corp.) diluted 1:10,000 was used for detection of the anti-TNF-α polyclonal serum antibodies. Whey samples were diluted 1:1 in wash buffer prior to assaying. Recombinant ovine IL-1β (gift of CSIRO Livestock Industries, Victoria, Australia) was diluted to generate a standard curve. The concentration of IL-1β was calculated by extrapolating from a standard curve and expressed in nanograms per milliliter.

ELISA for IL-10 and IL-12

Antibodies used to measure milk concentrations of IL-10 and IL-12 were described previously (Kwong et al., 2002; Hope et al., 2002). Plates were coated with either 4 μg/mL of mouse anti-bovine IL-10 (CC-318) or IL-12 (CC-301). Undiluted or 1:5 diluted whey samples were used in the ELISA for IL-12 and IL-10, respectively. Biotin-conjugated mouse anti-bovine IL-10 (CC-320) or IL-12 (CC-326) antibodies diluted to 1 or 8 μg/mL, respectively, were used for detection of the captured antigen. HRP-conjugated streptavidin (Sigma Chemical Co.) diluted 1:500 was used to detect the biotin-labeled antibodies. A standard curve was generated from supernatants derived from COS-7 cells transfected with cDNA encoding either IL-10 or IL-12. The biological activity of these IL-10 and IL-12 containing supernatants was assayed as previously described by the ability to inhibit or promote IFN-γ production, respectively (Kwong et al., 2002; Hope et al., 2002). The concentration of the supernatants were expressed in biological units of activity per milliliter and used to generate a linear standard curve. The concentrations of milk IL-10 and IL-12 were calculated by extrapolating from the respective standard curves of known biologically active known amounts of IL-10 and IL-12, and the milk values expressed in biological units of activity per milliliter. The intraassay coefficients of variation for 6 measurements of a whey sample assayed on the same ELISA plate were 9.9 and 6.2% for the IL-10 and IL-12 ELISA, respectively. The interassay coefficients of variation for 4 distinct measurements of a sample assayed on different plates was 11.5 and 9.3% for the IL-10 and IL-12 ELISA, respectively.

ELISA for C5a

The ELISA used to measure milk levels of C5a has been previously described (Shuster et al., 1997; Rainard et al., 1998), and performed here with only slight modification. Concentrations of milk C5a were measured by sandwich ELISA using anti-bovine C5a monoclonal antibody for antigen capture and rabbit anti-bovine C5a antiserum for detection (generous gifts of P. Rainard; Laboratoire de Pathologie Infectieuse et Immunologie, Institut National de la Recherche Agronomique, Nouzilly, France). Plates were coated with goat anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc., West Grave, PA) diluted to 5 μg/mL. The plates were washed, blocked, and subsequently incubated with anti-C5a monoclonal antibody (clone 6G4) diluted 1:5,000. Following a 1-h incubation at 37°C, plates were washed, and 100 μL of whey was diluted 1:10 in 0.05% Tween 20-TBS containing 0.1% gelatin, and 1 m*M* EDTA was added to each well. Plates were incubated for 1.5 h at room temperature and washed, and 100 μL of rabbit anti-bovine C5a/C5 diluted 1:2,500 in 0.05% Tween 20-TBS containing 0.1% gelatin was added to each well. Plates were incubated for 30 min at 37°C, washed, and 100 μL of goat anti-rabbit IgG conjugated to HRP (1:5,000 dilution in 0.05% Tween 20-TBS containing 0.1% gelatin) was added to each well. Following a 30-min incubation at 37°C, plates were washed, developed, and read as above. Milk levels of C5a, expressed in nanograms per milliliter, were calculated from a standard curve of known amounts of purified C5a-des-Arg.

ELISA for BSA, IL-8, IFN-γ, sCD14, and LBP

The ELISA for BSA, IL-8, sCD14, and LBP were all performed as previously described (Bannerman et al., 2003). A semi-quantitative, commercially available kit was used to measure bovine IFN-γ in undiluted whey samples according to the manufacturer's instructions (Biosource International, Inc., Camarillo, CA). The relative amounts of bovine IFN-γ were expressed in optical density (**OD**) units.

Statistical Methods

Repeated measures ANOVA with the Dunnett post hoc comparison test was performed using the PROC MIXED model (SAS 8.2; SAS Institute, Cary, NC) to compare the mean responses between experimental groups and the preinfused (time 0) groups. A paired *t*-test (GraphPad Prism 4.0; GraphPad Software Inc., San Diego, CA) was used to compare the mean maximal responses between quarters infused with saline and those infected with *K. pneumoniae*. For statistical analysis of milk SCC, data were transformed to $\log_{10}$ values. A P-value < 0.05 was considered significant.

RESULTS

Intramammary Challenge with *K. pneumoniae* Induces Both a Local and Systemic Inflammatory Response

Within 8 h of infusion, *K. pneumoniae* was recovered from all 7 infected quarters, and by 12 h the bacterial

load in 6 of the 7 infected quarters exceeded 1×10^5 cfu/mL (data not shown). At all time points >8 h, viable *K. pneumoniae* was recovered from milk samples obtained from all of the infected quarters. Saline-infused quarters remained free of detectable pathogens throughout the study. Clinical signs of mastitis were observed in all infected quarters within 20 h of challenge and included udder swelling and abnormal milk, characterized by the presence of clots and flakes, as well as a colorimetric change of the milk from white to yellow. Daily milk weights for each cow dropped by ~60% on the day following *K. pneumoniae* challenge (d 1) and further declined to ~15% of total prechallenge production by d 2 (Figure 1A). Milk output remained significantly depressed throughout the remainder of the study. Rectal temperatures increased within 12 h of challenge and reached a peak of 40.7 + 0.28°C by 16 h (Figure 1B). Rectal temperatures remained elevated up to 20 h postchallenge. The ability of intramammary infusion of *K. pneumoniae* to elicit a systemic response was further demonstrated by an increase in plasma levels of the hepatically derived acute-phase response protein, LBP (Figure 2). Circulating levels of LBP increased within 24 h after challenge and remained elevated throughout the study. Peak levels of plasma LBP were observed at 48 and 72 h postchallenge and reached a maximal concentration of 179.5 + 9.6 μg/mL.

Systemic involvement following IMI with *K. pneumoniae* was further evidenced by a significant drop in the number of circulating neutrophils. Circulating levels of neutrophils reached a minimum of 755 + 260 cells/μL within 16 h and remained below preinfection levels for up to 48 h postchallenge (Figure 3A). The decrease in circulating neutrophils paralleled an increase in milk somatic cells in quarters challenged with *K. pneumoniae* (Figure 3B). Milk SCC (**MSCC**) in challenged quarters increased within 16 h of challenge and continued to increase throughout the study, reaching a maximum of $51.5 \times 10^6 + 18.9 \times 10^6$ cells/mL at 96 h. In contrast to the infected quarters, there was no significant change in MSCC in quarters receiving saline. At 96 h, MSCC in saline control quarters approached, but did not reach a level that was significantly different from the time 0 ($P = 0.062$).

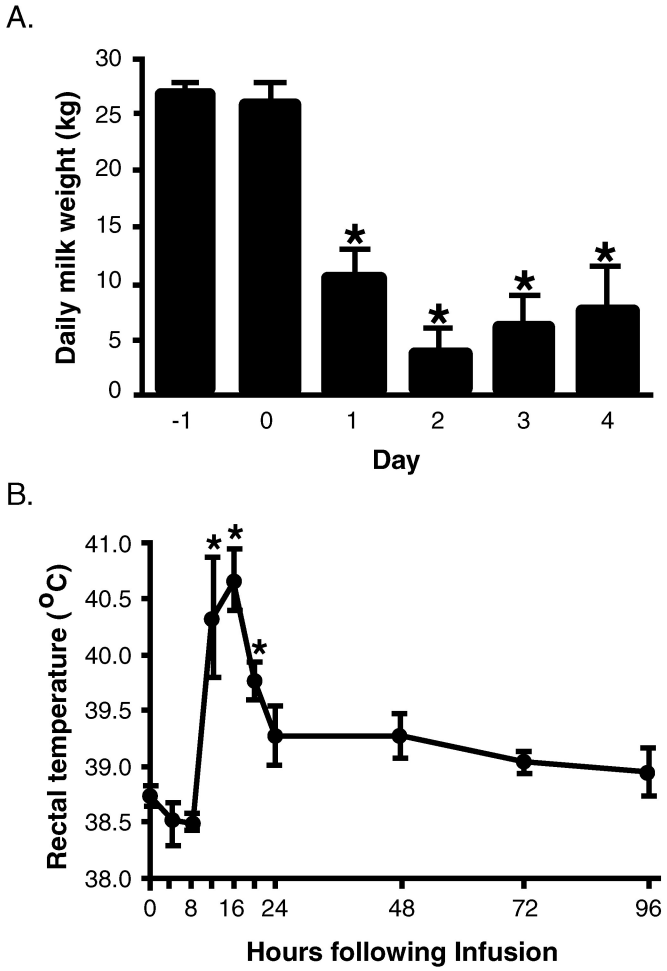

Figure 1. Effect of intramammary *Klebsiella pneumoniae* infection on daily milk weights and temperature. Total milk weight (sum of morning and evening outputs) data were collected 1 d prior to (–1), d 0, and for 4 d following *K. pneumoniae* challenge (A). The vertical bars represent the mean + SE of milk weights in kilograms. *Significantly decreased compared to d 0 ($P < 0.05$). As an indicator of the systemic response, rectal temperatures were measured immediately prior to and for various time points following IMI (B). Mean (+ SE) temperature is reported in °C. *Significantly increased compared to time 0 ($P < 0.05$).

Intramammary Infection with *K. pneumoniae* Elicits a Breakdown in the Integrity of the Blood-Mammary Gland Barrier

To determine whether changes in the permeability of the mammary gland vasculature occur following infection with *K. pneumoniae*, milk BSA levels were assayed by ELISA. Milk obtained from infected quarters showed a marked increase in BSA levels within 12 h of challenge (Figure 4). Within 20 h, the milk BSA levels in infected quarters were >40-fold higher than the levels in saline control quarters. The augmented levels of milk BSA in *K. pneumoniae* infused quarters persisted throughout the study. In contrast to infected quarters, there was no significant change in milk BSA levels in saline control quarters throughout the first 24 h. At later time points, however, there was a significant increase in milk BSA levels in these quarters relative to those at time 0 (preinfusion). Peak levels of BSA in infected quarters (1.14 + 0.03 mg/mL) were significantly higher than those reached in quarters receiving saline alone (0.55 + 0.14 mg/mL).

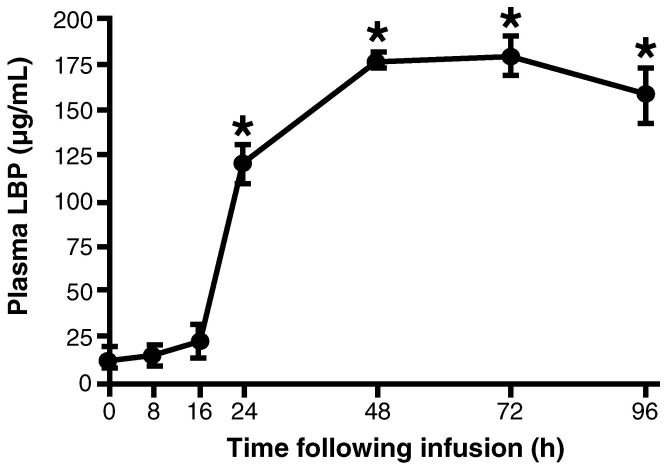

Figure 2. Intramammary infection with *Klebsiella pneumoniae* increases circulating levels of the acute-phase response protein, LPS-binding protein (LBP). Blood samples were collected immediately prior to and for various time points following IMI and plasma assayed for LBP by ELISA. Mean (+SE) levels of plasma LBP are reported in μg/mL. *Significantly increased compared to time 0 ($P < 0.05$).

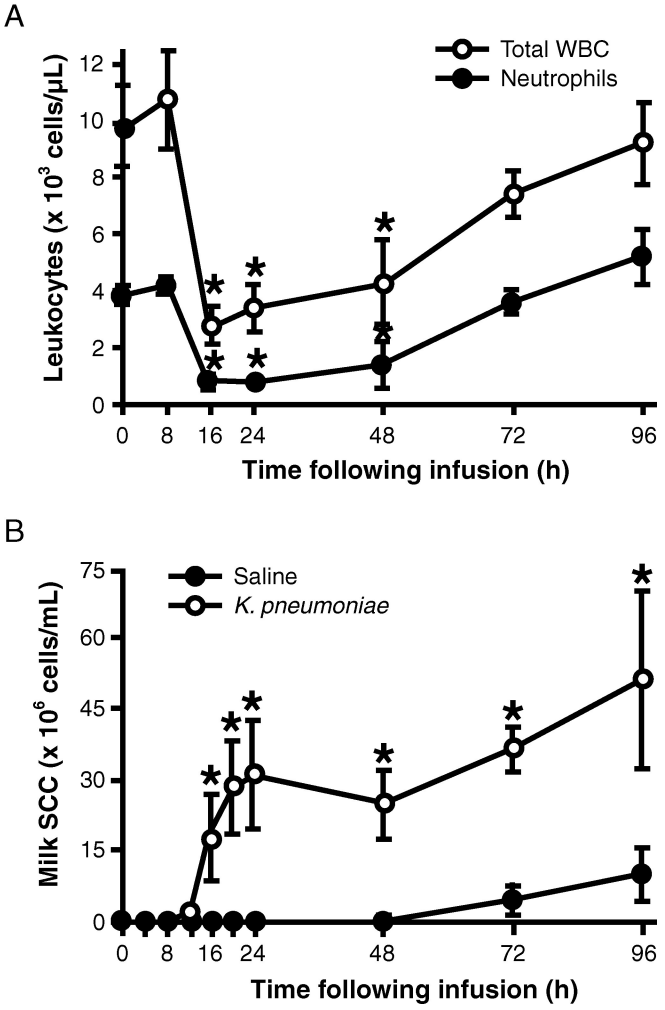

Figure 3. Effect of IMI with *Klebsiella pneumoniae* on circulating white blood cells and neutrophils and milk SCC. Total white blood cell (WBC) and differential neutrophil counts were determined in blood obtained from the tail veins of cows immediately prior to and for various time points following IMI (A). Mean (+SE) cell counts are reported in thousands/μL. *Significantly decreased compared with time 0 ($P < 0.05$). In addition to blood collection from the tail vein, milk samples were obtained from saline and *K. pneumoniae* infected quarters and milk somatic cells counted (B). Mean (+SE) milk SCC are reported in millions/mL. *Significantly increased compared to time 0 ($P < 0.05$).

Increased Levels of Endogenous Chemotactic Agents in Milk Following Intramammary Challenge with *K. pneumoniae*

The IL-8 and C5a are chemotactic agents that facilitate neutrophil recruitment to the site of infection. Increased levels of both of these agents were observed in infected quarters within 16 h of challenge (Figure 5). Maximal levels of IL-8 (715 + 58 pg/mL) and C5a (58.2 + 15.2 ng/mL) were observed in the milk of infected quarters within 16 and 20 h, respectively. Relative to preinfused quarters, elevated levels of milk IL-8 were observed in infected quarters until 48 h postchallenge (Figure 5A), whereas increased levels of milk C5a were detected in infected quarters until the end of the study (Figure 5B). There was no change in C5a or IL-8 levels in milk obtained from saline control quarters at any time points assayed.

K. pneumoniae Infection Elicits Both a Pro- and Antiinflammatory Cytokine Response in the Mammary Gland

The TNF-α and IL-1β are proinflammatory cytokines that contribute to host defense mechanisms during infection. To determine whether infection with *K. pneumoniae* changes the expression of these cytokines in the mammary gland, milk levels of TNF-α and IL-1β were assayed by ELISA (Figure 6). Initial increments in TNF-α levels in the milk of infected quarters were evident within 12 h of infection and elevated levels were maintained for an additional 60 h (Figure 6A). In contrast, there were no detectable levels of TNF-α in milk obtained from quarters infused with saline alone at any time points throughout the study. Increases in IL-1β were evident in *K. pneumoniae*-infected quarters >20 h postchallenge (Figure 6B). Surprisingly, increases in IL-1β were also observed in saline control quarters at >24 h following infusion.

The IL-12 contributes to host defense mechanisms by: 1) upregulating IFN-γ production, the latter of which serves to prime leukocytes, and 2) promoting T-cell proliferation and differentiation, thus, serving as a link

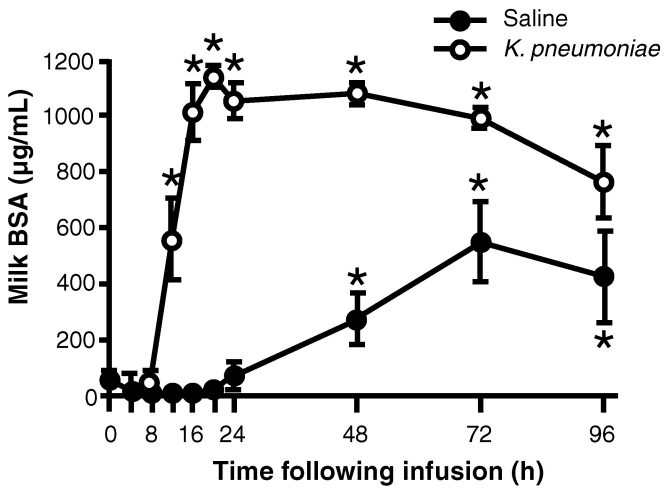

Figure 4. Intramammary infection with *Klebsiella pneumoniae* increases mammary vascular permeability. As a marker of the integrity of the blood-mammary gland barrier, milk BSA levels were assayed by ELISA. Milk samples were obtained from quarters immediately prior to and for varying time points following saline or *K. pneumoniae* infusion. Mean (+SE) BSA levels are reported in μg/mL. *Significantly increased compared to levels in preinfused (time 0) quarters ($P < 0.05$).

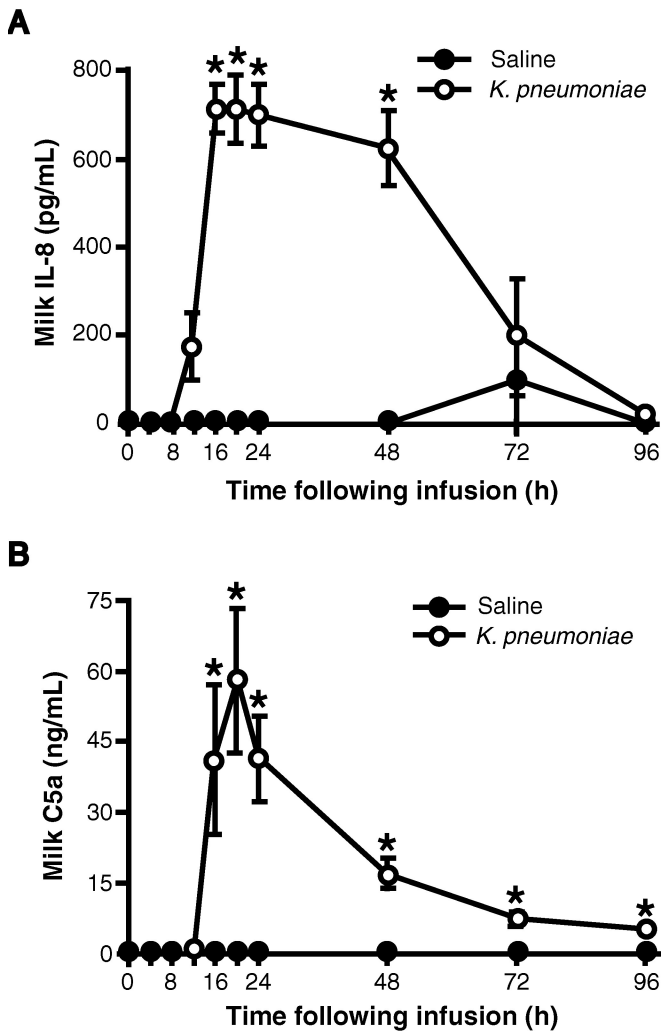

Figure 5. Effect of IMI with *Klebsiella pneumoniae* on IL-8 and C5a levels in milk. Levels of IL-8 (A) and C5a (B) in milk obtained from quarters infused with saline or *K. pneumoniae* were quantified by ELISA. Mean (+SE) IL-8 and C5a concentrations are reported in picograms and nanograms per milliliter, respectively. *Significantly increased compared to levels in preinfused (time 0) quarters ($P < 0.05$).

between the innate and adaptive immune systems. Increased levels of milk IL-12 were detectable within 16 h of infection and peaked 32 h later (Figure 7A). The IL-12 levels remained elevated in infected quarters throughout the end of the study. Because IL-12 is known to promote IFN-γ production, milk levels of the latter were similarly assayed (Figure 7B). Initial increases in IFN-γ levels in infected quarters, relative to those in prechallenged quarters, were evident within 12 h of challenge, and elevated levels were maintained throughout the study period. Interestingly, there was a significant increase in IFN-γ levels in saline control quarters at >48 h; however, peak levels were significantly lower than those in *K. pneumoniae* infected quarters.

Down-regulation of the proinflammatory response is a key requisite to the resolution of infection and is mediated, in part, by IL-10. Increased levels of milk IL-10 were detected in infected quarters within 20 h of infection, peaked at 48 h, and remained elevated relative to time 0 levels throughout the study (Figure 7C). In contrast to the detectable increase in IL-10 and IL-12 following *K. pneumoniae* infection, these cytokines were undetectable in quarters receiving saline alone at all time points assayed (Figure 7).

Intramammary Infection with *K. pneumoniae* Increases Milk Levels of sCD14 and LBP

The sCD14 and LBP are key accessory molecules that enable both host recognition and neutralization of LPS, a proinflammatory constituent of the outer membrane of gram-negative bacteria. To determine whether *K. pneumoniae* infection could alter mammary gland levels of sCD14 and LBP, milk levels of these molecules were quantified by ELISA (Figure 8). Relative to prechallenged quarters, increased levels of milk sCD14 were detected in infected quarters as early as 20 h postinfection and persisted throughout the study (Figure 8A). Significantly higher levels of LBP were detected in *K. pneumoniae* challenged quarters as early as 16 h postchallenge and persisted throughout the study (Figure 8B). Interestingly, elevated levels of milk sCD14 and LBP were detected in saline control quarters at >20 and 48 h, respectively. Maximal levels of sCD14

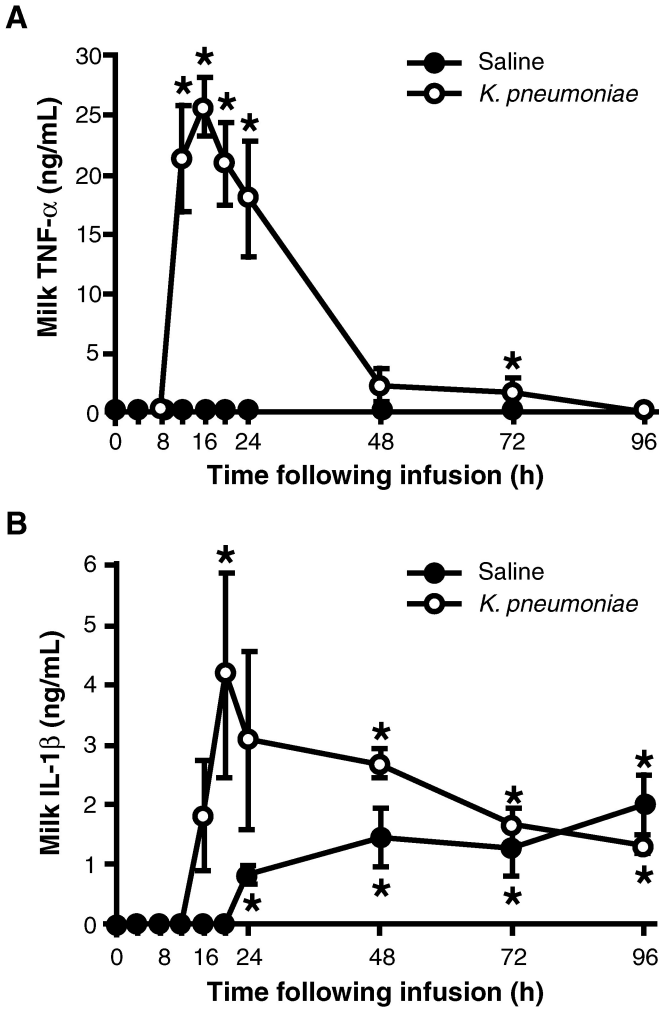

Figure 6. Effect of *Klebsiella pneumoniae* infection on TNF-α and IL-1β levels in milk. ELISA were used to determine the concentrations of TNF-α (A) and IL-1β (B) in milk obtained from quarters infused with saline or *K. pneumoniae*. Mean (+SE) TNF-α and IL-1β concentrations are reported in ng/mL. *Significantly increased compared to levels in preinfused (time 0) quarters ($P < 0.05$).

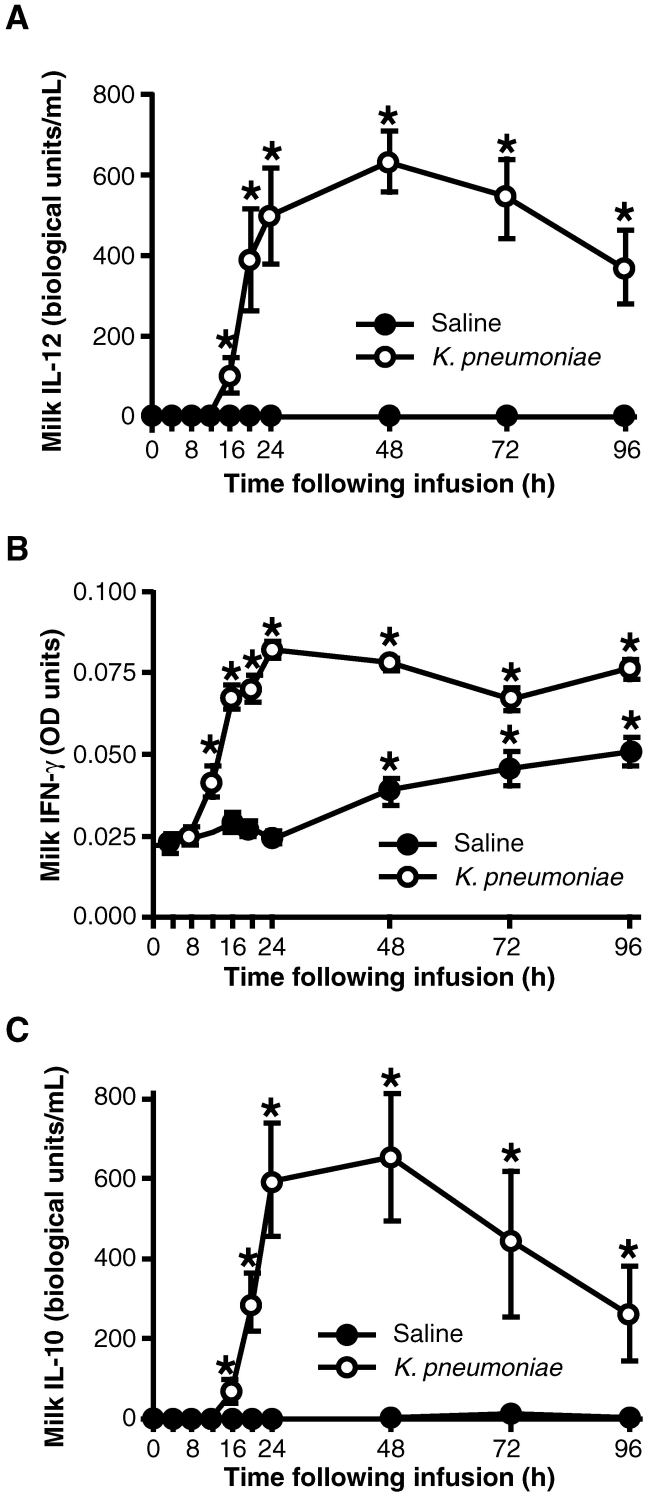

Figure 7. Intramammary challenge with *Klebsiella pneumoniae* increases milk levels of IL-12, IFN-γ, and IL-10. Levels of IL-12 (A), IFN-γ (B), and IL-10 in milk obtained from quarters infused with saline or *K. pneumoniae* were quantified by ELISA. Mean (+SE) IL-12 and IL-10 levels are reported in biological units of activity/mL, whereas IFN-γ concentrations are reported in optical density (OD) units. *Significantly increased compared to levels in preinfused (time 0) quarters ($P < 0.05$).

(38.74 + 8.05 μg/mL vs. 19.80 + 7.06 μg/mL), but not LBP (49.93 + 0.51 μg/mL vs. 41.97 + 6.09 μg/mL) in infected quarters were significantly higher than the peak levels reached in the saline control quarters.

DISCUSSION

The ability of bacteria to establish infection is mediated by both intrinsic properties of the pathogen, itself, and the ability of the host to respond to the invading organism (Burvenich et al., 2003). The innate immune system represents the first line of defense in the host response to infection (Hoffmann et al., 1999). Unlike the adaptive immune response, which requires a prolonged infection-to-response lag time, the innate immune sys-

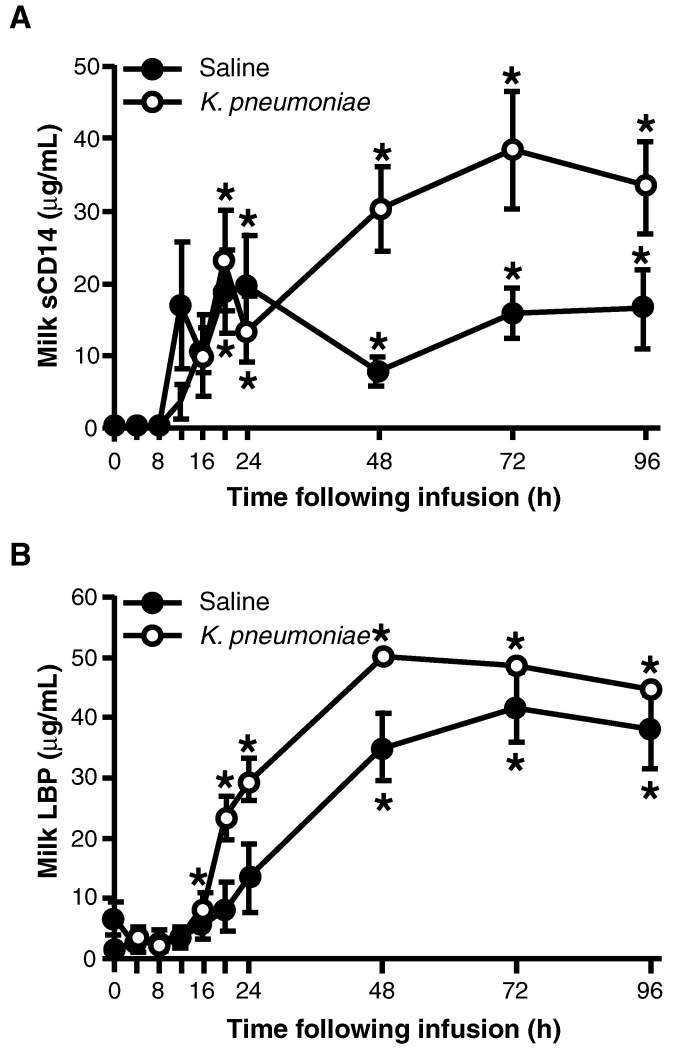

Figure 8. Intramammary challenge with *Klebsiella pneumoniae* increases milk levels of sCD14 and LBP. Levels of sCD14 (A) and LBP (B) in milk obtained from quarters infused with either saline or *K. pneumoniae* were assayed by ELISA. *Significantly increased compared to levels in preinfused (time 0) quarters ($P < 0.05$).

tem is poised to immediately recognize and respond to the earliest stages of infection. Although the innate immune system has evolved to recognize the multitudes of pathogens that exist in nature, the response varies depending on the organism. For example, IMI with *E. coli*, but not *Staphylococcus aureus*, induces the upregulation of TNF-α, IL-1β, and IL-8 (Riollet et al., 2000). These differences in the host innate immune response may contribute to the limited acute IMI caused by *E. coli* and the more chronic infectious state characteristic of *Staphylococcus aureus*-induced mastitis. Even among the gram-negative organisms that induce mastitis, including *E. coli*, *Serratia marcescens*, and *K. pneumoniae*, differences in the clinical course and duration of infection have been reported (Hazlett et al., 1984; Todhunter et al., 1990; Todhunter et al., 1991). Because much of the understanding of the innate immune response to gram-negative infection has been derived from experiments using *E. coli* as the model organism, the present studies examined the host response to another clinically relevant gram-negative pathogen, *K. pneumoniae*.

The TNF-α and IL-1β are proinflammatory cytokines produced by cells of monocytic lineage and neutrophils and are potent inducers of fever and acute-phase protein synthesis (Dinarello, 1996). Within 20 h of *K. pneumoniae* challenge, elevated levels of both TNF-α and IL-1β were detected in milk from infected quarters (Figure 6), and were temporally coincident with following *K. pneumoniae* challenge, whereas maximal levels of TNF-α following *E. coli* infection have been reported to range from 3.25 + 0.8 ng/mL (Lee et al., 2003) to 14.1 + 3.2 2.4 ng/mL increased body temperature (Figure 1). Peak milk TNF-α levels were observed between 12 to 24 h postinfection, a time frame in which increased circulating levels of the acute-phase protein, LBP, were detected. The initial temporal increase in milk TNF-α levels within 12 to 16 h of *K. pneumoniae* infection followed by a dramatic decline at >24 h are comparable with similar changes in TNF-α levels reported following *E. coli* infection (Shuster et al., 1997; Riollet et al., 2000; Lee et al., 2003). The TNF-α levels reached a peak concentration of 25.68 + ng/mL (Riollet et al., 2000). Maximal amounts of IL-1β (4.17 + 1.7 ng/mL) were observed at 20 h postchallenge, and both the amount of IL-1β produced and the temporal increase in the level of this cytokine are consistent with prior studies that have used *E. coli* as the challenge organism (Shuster et al., 1995; Shuster et al., 1997).

Decreases in circulating neutrophils were observed within 16 h and temporally coincident with increases in MSCC in infected quarters (Figure 3). Because neutrophils can constitute >90% of the milk somatic cells present during mastitis (Saad and Ostensson, 1990), these data are consistent with circulating neutrophil recruitment to the infected quarter. Initial increases in MSCC at 16 h following infection paralleled initial elevations in the chemoattractants IL-8 and C5a (Figure 5). Elevated levels of another chemoattractant, leukotriene B_4 (LTB_4), have been reported following IMI with *K. pneumoniae* (Rose et al., 1989). Thus, the combined presence of IL-8, C5a, and LTB_4 provides a chemotactic-rich environment that would be expected to promote neutrophil recruitment. Whether any one of these chemoattractants serves as the primary agent responsible for neutrophil recruitment remains unknown. In the previous study showing an increase in LTB_4 following *K. pneumoniae* infection, initial in-

creases in MSCC preceded detectable increases in LTB_4 levels (Rose et al., 1989). Whether earlier increases in LTB_4 were not observed due to detection limits of the assay or whether initial increases in MSCC occurred independent of LTB4 remains unclear. Because the infectious inoculum used in that study (10^7 cfu/quarter) was so much higher than that used here (10^2 cfu/quarter), it is difficult to compare temporal changes in identical parameters, such as increases in MSCC, between the 2 studies.

Increased levels of milk BSA, an indicator of increased mammary vascular permeability, were observed within 12 h of challenge and remained elevated throughout the study (Figure 4). This temporal increase and sustained elevation of BSA is comparable to measured changes in BSA following *E. coli* infection (Riollet et al., 2000). The initial detection and subsequent increases in milk concentrations of C5a paralleled maximal increases in milk levels of BSA between 16 and 24 h postinfection (Figure 4). Because the level of complement in milk from healthy animals is relatively low (Rainard and Poutrel, 1995), these findings are consistent with a serum-derived influx of complement into the mammary gland during breakdown of the blood-milk barrier, and the subsequent activation of complement leading to the generation of detectable levels of complement fragments.

In comparison to TNF-α and IL-8, peak amounts of IL-10 and IL-12 were observed at later time points (Figure 7). Maximal levels of both cytokines were observed 48 h postinfection and remained elevated throughout the end of the study. Increases in milk IL-10 concentrations occurred concomitant with decreases in TNF-α. This latter finding is consistent with reports that IL-10 inhibits TNF-α production (Cassatella et al., 1993; Armstrong et al., 1996). Increases in IFN-γ closely paralleled those of IL-12, the latter of which promotes IFN-γ production (Collins et al., 1998; Munder et al., 1998). Further, in a positive-feedback manner, IFN-γ is able to induce the upregulation of IL-12 (Ma, 2001). Thus, the comparable rise in concentrations of IFN-γ and IL-12 over time is consistent with the ability of each cytokine to stimulate the production of the other. These data, however, do not preclude a role for other cytokines in mediating the induction of either of the cytokines as well.

The accessory molecules sCD14 and LBP contribute to the induction of an inflammatory response to gram-negative infection by facilitating Tlr-4 recognition of LPS (Guha and Mackman, 2001). The sCD14 is derived from monocytes by direct exocytosis and from proteolytic cleavage of mCD14 on the cell surface (Viriyakosol and Kirkland, 1995), whereas LBP is primarily synthesized by hepatocytes, and its expression is greatly upregulated during the acute-phase response to bacterial infection (Tobias et al., 1999; Schumann and Latz, 2000). Although LPS can directly bind to CD14, LBP greatly enhances this process by dissociating LPS aggregates into LPS monomers and transferring these monomers to CD14 (Hailman et al., 1994).

Several studies using different experimental approaches, including the use of $LBP^{-/-}$ or $CD14^{-/-}$ mice, CD14 neutralizing antibodies, and administration of exogenous LBP, have established a protective role for CD14 and LBP in mediating the host response to LPS and gram-negative bacterial infection (Jack et al., 1997; Lamping et al., 1998; Le Roy et al., 2001; Wenneras et al., 2001; Yang et al., 2002; Fierer et al., 2002). In fact, a recent investigation demonstrated that intramammary coadministration of sCD14 with an inoculum of *E. coli* enhanced bacterial clearance, thereby implicating a potential role for sCD14 in mediating bovine mammary gland response to infection (Lee et al., 2003). Other studies have reported that the impaired ability of the host to fight gram-negative infection when levels of sCD14 or LBP are depressed correlates with decreased proinflammatory cytokine and chemokine production (Le Roy et al., 2001; Yang et al., 2002; Fierer et al., 2002), as well as decreased neutrophil responsiveness (Jack et al., 1997; Le Roy et al., 2001; Yang et al., 2002; Fierer et al., 2002). In addition to contributing to host cell recognition, sCD14 and LBP facilitate the transfer of LPS to lipoproteins, rendering LPS incapable of eliciting an inflammatory response (Wurfel et al., 1995). Thus, although sCD14 and LBP contribute to the host cell recognition of LPS and the subsequent generation of proinflammatory mediators, these molecules also serve to moderate inflammation and prevent a response that may become excessive and deleterious to the host itself.

Intramammary challenge with *K. pneumoniae* induced a significant increase in both sCD14 and LBP concentrations in infected quarters. Peak levels of both proteins were detected between 48 and 72 h following infection (Figure 8). Because LBP and sCD14 act in concert to facilitate host recognition and/or neutralization of LPS, the simultaneous increase of both of these proteins would be advantageous to the host. Increases in milk TNF-α concentrations occurred prior to increases in either LBP or sCD14, suggesting that the elicitation of initial proinflammatory responses can occur in the presence of basal levels of sCD14 and LBP. Interestingly, peak levels of MSCC, IL-10, IL-12, and IFN-γ production were all observed at times of maximal elevation of both sCD14 and LBP. Whether increments in sCD14 and LBP are necessary for maximal neutrophil recruitment and elevation of these cytokines remains unknown. Because sCD14 and LBP enhance

LPS-induced neutrophil adhesion and priming of neutrophil superoxide release (Shapira et al., 1995; Hailman et al., 1996; Troelstra et al., 1997), elevated levels of sCD14 and LBP in the gland at the time of maximal neutrophil recruitment may serve to potentiate the ability of these cells to control infection.

During the acute-phase response to infection, the proinflammatory cytokines TNF-α, IL-1β, and IL-6 stimulate hepatocyte synthesis of LBP (Schumann et al., 1994). Following *K. pneumoniae* challenge, increased levels of circulating LBP were detected within 8 h of peak increases in body temperature and TNF-α production, both of which are hallmarks of the acute-phase response. Increases in the concentrations of LBP in milk occurred at a time when circulating levels of LBP were increased and at a time of increased mammary vascular permeability, the latter of which was reflected by an increase in milk BSA levels. Together, these data suggest that the increased levels of LBP in milk resulted from vascular leak of LBP from the circulation into the mammary gland.

Interestingly, a slight increase in milk SCC and more dramatic increases in milk BSA and LBP concentrations were detected in saline control quarters towards the end of the study. These changes occurred in the absence of detectable infection and complement activation. A significant increase in IL-1β and IFN-γ levels was initially detected in these quarters between 24 and 48 h, respectively; however, there was no detectable production of the other proinflammatory cytokines assayed, including IL-8, TNF-α, or IL-12. Previous studies on *K. pneumoniae*-induced mastitis have reported ~ 5- (Kunkel et al., 1987) and 10-fold (Rose et al., 1989) increases in MSCC and a >60% increase in milk BSA levels (Rose et al., 1989) in control quarters. Because this elevation of milk BSA reflects increased vascular permeability and that permeability changes in the control quarters occurred at a time when circulating levels of LBP were augmented, the detection of elevated levels of LBP in control quarters is consistent with LBP leakage from the vasculature into control quarters.

As mentioned previously, there are differences in the clinical course and duration of infection following IMI with either *E. coli* or *K. pneumoniae*. Despite these differences, there is a striking similarity in the host innate immune responses to these bacteria. Similarities in the kinetics and overall response to *K. pneumoniae*, which are reported here, and to *E. coli*, which have been published previously, include: 1) peak induction of a proinflammatory cytokine response, characterized by the production of IL-1β, IL-8, and TNF-α within 24 h of infection (Riollet et al., 2000; Lee et al., 2003); 2) early breakdown of the blood-mammary gland barrier characterized by increased milk levels of BSA (Riollet et al., 2000); and 3) activation of complement within 16 h of challenge (Riollet et al., 2000). Other parameters, such as changes in sCD14, LBP, IL-10, and IL-12 have yet to be reported following infection with *E. coli*. In contrast to experimental infection with gram-negative organisms, IMI with the Gram-positive organism *Staphylococcus aureus* is characterized by a lack of induction of a proinflammatory cytokine response and relatively weak activation of milk complement proteins (Riollet et al., 2000). A common characteristic of gram-negative, but not Gram-positive bacteria, is the presence of the highly proinflammatory component LPS. Similar to infection with either *E. coli* or *K. pneumoniae*, LPS induces all of the innate immune responses mentioned above, but with more rapid kinetics (Bannerman et al., 2003). Thus, *E. coli* and *K. pneumonia*, as well as LPS are able to elicit a qualitatively similar innate immune response.

The present study is the first to detail the proinflammatory cytokine response elicited by IMI with *K. pneumonia* and to report on changes in mammary gland levels of LBP during the course of infection. To our knowledge, this is also the first report to directly measure the levels of IL-10 and IL-12 in milk during IMI. Based on the present findings, we conclude that *K. pneumonia* is capable of eliciting a highly proinflammatory state that consists of intramammary elevations of proinflammatory cytokines and chemotactic molecules, massive influx of circulating neutrophils, and increased levels of accessory molecules involved in LPS recognition.

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